Wanderings in Biochemistry

Peter Lengyel

From the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520

My Ph.D. thesis in the laboratory of Severo Ochoa at New York University School of Medicine in 1962 included the determination of the nucleotide compositions of codons specifying amino acids. The experiments were based on the use of random copolyribonucleotides (synthesized by polynucleotide phosphorylase) as messenger RNA in a cell-free protein-synthesizing system. At Yale University, where I joined the faculty, my co-workers and I first studied the mechanisms of protein synthesis. Thereafter, we explored the interferons (IFNs), which were discovered as antiviral defense agents but were revealed to be components of a highly complex multifunctional system. We isolated pure IFNs and characterized IFN-activated genes, the proteins they encode, and their functions. We concentrated on a cluster of IFN-activated genes, the p200 cluster, which arose by repeated gene duplications and which encodes a large family of highly multifunctional proteins. For example, the murine protein p204 can be activated in numerous tissues by distinct transcription factors. It modulates cell proliferation and the differentiation of a variety of tissues by binding to many proteins. p204 also inhibits the activities of wild-type Ras proteins and Ras oncoproteins.

From Budapest to the Ochoa Laboratory in New York

I was born in Budapest, Hungary, in 1929. As an adolescent, I wanted to study medicine, following in my grandfather's footsteps. It was he, an internist who obtained his medical training in the 1890s, who changed my mind. He was frustrated by the huge gaps in the scientific basis of medicine in the 1940s, and he advised me to study organic chemistry and biochemistry and apply what I have learned to biomedical research.

Thus, I matriculated at the Technical University of Budapest and graduated in 1951 as a chemical engineer. After serving in the Hungarian army for two years, I sought admission to graduate school to study biochemistry. For this, I had to request a letter of recommendation from my professor of organic chemistry at the Technical University. Learning my career choice, the professor visibly saddened and asked, "Do you really want to spend your whole life studying proteins?" At the time, this was a reasonable question. Organic chemistry was already a well developed field, biochemistry was way behind, and molecular biology was nonexistent. That DNA was a carrier of hereditary information had been discovered only in 1944, and this discovery was still doubted by many. It was only later, in 1951, that the amino acid sequence of the first protein, the 30-amino acid-long B-chain of insulin, was determined and found to be unique by Sanger and Tuppy (1). Earlier, it had been frequently suggested that proteins may not be pure chemical entities but may consist of mixtures of closely related substances. Despite these uncertainties in the field of biochemistry at the time, I received the letter of recommendation and was accepted as a graduate student.

My advisor became F. B. Straub, professor of biochemistry at the Medical University of Budapest. He was a student of Albert Szent-Györgyi, a Hungarian biochemist who was awarded the Nobel Prize for various contributions, including the discovery of vitamin C. Dr. Straub was a gifted biochemist, whose contributions included the purification and crystallization of malic and lactic
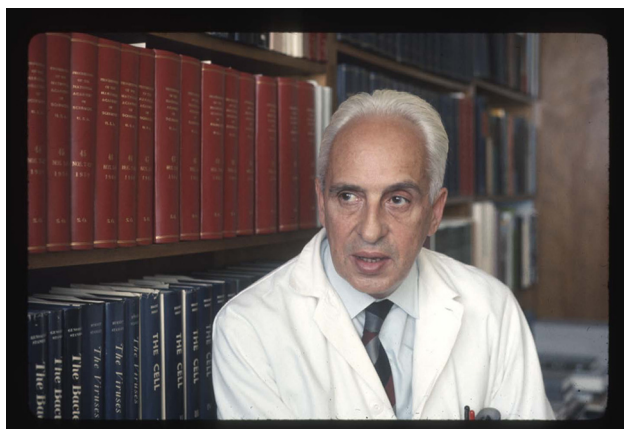
FIGURE 1. **Severo Ochoa (New York City, circa 1970).**

dehydrogenases and the discoveries of diaphorase and the muscle protein actin (2). During the two and a half years I spent with Dr. Straub, I received my introduction to biochemistry, including a warning: before starting a research project, you must decide that you consider it worthwhile doing. One of the textbooks I used in preparation for the qualifying examination was by Fruton and Simmonds, two professors of biochemistry at Yale University.

After the collapse of the Hungarian uprising against the Soviet oppression in 1956, I fled to the West, together with 200,000 Hungarians. I immigrated to the United States, which I had considered the center of biochemical research. While still in Hungary, I read with great interest about the discovery of the first enzyme capable of synthesizing RNA in the test tube: polynucleotide phosphorylase (PNP) by Grunberg-Manago and Ochoa at New York University (NYU) School of Medicine (3). Upon arriving in New York, I approached Dr. Ochoa. It must have been his sympathy for the cause of Hungary and my enthusiasm to become his student that induced him to accept me as a graduate student in his laboratory.

Dr. Ochoa made great contributions to various fields in biochemistry, including the Krebs cycle, fatty acid synthesis, oxidative phosphorylation, propionate metabolism, and peptide chain initiation (Fig. 1) (4). He was awarded the Nobel Prize in 1959 for the discovery of PNP. He shared the prize with his former postdoctoral associate, Arthur Kornberg, who obtained it for discovering DNA polymerase.

The Ochoa laboratory was a great place to learn. Lunches and coffee breaks were taken together. These were the times for discussing recent publications, results of experiments, and new ideas. My innate interest in these discussions prompted me to follow the literature diligently.

The faculty member at NYU from whom I learned very much was Charlie Gilvarg. He knew everything that was worthwhile knowing. I consulted him often and missed him after his move from NYU to Princeton University. The long-standing friendship among Yoshito Kaziro, Charles Weissmann, and me started in the Ochoa laboratory. Both have had more distinguished careers than I have had.

An important chapter of my research career resulted from my studies as a graduate student with Dr. Ochoa. My first assignment in his laboratory was to study the degradation of various natural RNAs by phosphorolysis catalyzed by PNP in the presence of inorganic phosphate. Thereafter, with R. W. Chambers, I established that PNP can also synthesize, from the appropriate ribonucleoside diphosphates, an RNA containing 2-thiouridylate (besides nucleotides A, C, G, and U) (5). Somewhat earlier, 2-thiouridylate was found to be present in certain natural RNAs.

My next project, suggested by Dr. Ochoa, was part of his exploration of propionic acid metabolism in animal tissues. The goal was to characterize methylmalonyl isomerase from sheep kidney. This enzyme converts methylmalonyl coenzyme A, a branched chain compound, to succinyl coenzyme A, its straight chain isomer. I noted the similarity of the above conversion to that of β-methylaspartate, another branched chain compound, to glutamate, its straight chain isomer, by glutamate isomerase, as reported by Barker *et al.* (6). Recognizing this similarity, I expected that methylmalonyl isomerase may require for activity the same vitamin B_{12} cobamide coenzymes as glutamate isomerase. In collaboration with R. Mazumder at NYU, I started to search for vitamin B_{12} coenzyme in our enzyme. However, prior to completing our study, three research papers reported finding vitamin B_{12} coenzyme in methylmalonyl isomerase. Although disappointed, we completed our study and found vitamin B_{12} coenzyme in our sheep kidney enzyme. We established that, of the three types of these coenzymes, only two, dimethylbenzimidazolylcobamide and benzimidazolylcobamide, appeared to fully activate the enzyme (7).

Thereafter, I was supposed to join an ongoing project concerning the DNA-dependent enzymatic synthesis of RNA. However, my participation in the project was cut short. This happened because, completely unexpectedly, I became involved in the initial deciphering of the genetic code.

This exciting and competitive study became the topic of my Ph.D. thesis in 1962. I recently described the detailed story of this undertaking and its origins in a prefatory chapter of the *Annual Review of Microbiology*. It is entitled

"Memories of a senior scientist: on passing the fiftieth anniversary of the beginning of deciphering the genetic code" (8). Thus, I will not present it here.

I will just note that the two laboratories involved in the deciphering of the nucleotide compositions of codons were those of Marshall Nirenberg at the National Institutes of Health and Dr. Ochoa at NYU. The competition between the two laboratories (called by some "the code war") accelerated the research (4, 8, 9).

My Ph.D. thesis was entitled "Use of synthetic polynucleotides in the deciphering of the genetic code." It included the findings in the first five articles from the series "Synthetic polynucleotides and the amino acid code." The first article by Lengyel *et al.* (10) described the preparation of random copolynucleotides produced by PNP and containing two or more kinds of nucleotides. These were translated in a cell-free amino acid-incorporating system to reveal the nucleotide compositions of codons. The five articles reported the correct nucleotide compositions of codons for 18 amino acids.

Besides the above, a short section of my thesis was based on the treatment of synthetic polyribonucleotides with nitrous acid. This revealed that the purine nucleotide I (inosinate, which contains hypoxanthine) has the same coding properties as guanylate, and the purine nucleotide X (xanthylate, which contains xanthine) is inactive in coding (11).

Another short section of my thesis identified the bacterial ribosome as a target of action of the antibiotic streptomycin (12). Earlier, Erdos and Ullmann (13) reported that this antibiotic inhibited the incorporation of labeled amino acids into an extract from streptomycin-sensitive but not streptomycin-resistant bacteria. Moreover, Spotts and Stanier (14) hypothesized that ribosomes from streptomycin-sensitive bacteria have a high affinity for streptomycin, whereas ribosomes from resistant strains have no affinity. To test their hypothesis, Speyer *et al.* (12) fractionated cell-free amino acid-incorporating systems from streptomycin-sensitive and streptomycin-resistant *Escherichia coli* into fast sedimenting ribosomal and slow sedimenting supernatant fractions. We established that when mixing one of the two types of ribosomes (streptomycin-sensitive or streptomycin-resistant) with one of the two types of supernatant fractions (streptomycin-sensitive or streptomycin-resistant), it was the ribosomal fraction (streptomycin-sensitive or streptomycin-resistant) that determined whether the mixture was sensitive or resistant to streptomycin (12). A later publication reported that several other antibiotics also impaired ribosome activity (15).
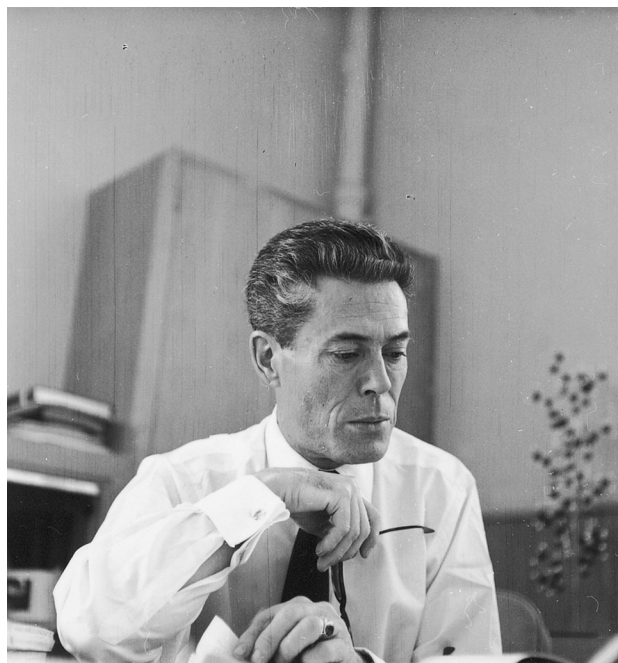

FIGURE 2. **Jacques Monod (Paris, 1964).**

A Year at the Pasteur Institute

I ended my involvement in the studies on the genetic code at NYU in the summer of 1963, and I spent one year at the Pasteur Institute in Paris in the laboratory of the brilliant French scientist Jacques Monod (16). At the time, the Pasteur Institute was among the prime movers of progress in molecular biology. Jaçques Monod and Francois Jacob were responsible for the discovery of regulatory genes, operators and repressors, operons, and messenger RNA and also contributed much to the understanding of allosteric regulators. Besides being a highly original and imaginative scientist, Monod was also a warm and generous man of great charm (Fig. 2) (16).

Just as in the Ochoa laboratory, lunch was eaten together in the Pasteur Institute. Scientific discussions were also just as lively, especially because the luminaries André Lwoff, Monod, and Jacob, who shared the Nobel Prize a few years later, actively participated (16). The difference was in the source of the meals. At NYU, it was the cafeteria. This was not so in the Pasteur Institute. Although a cafeteria had just opened and was serving a variety of dishes, as well as white and red wine for lunch, the French researchers found the choice too restricted, and most of them continued to obtain their food from the neighboring charcuterie. Another difference was in the hours worked. While at NYU, I often got home after midnight. At the Pasteur Institute, at the time (perhaps to save electricity), you had to leave before 8 p.m. To stay later, you needed special permission, which was valid for only

one evening. This had to be requested for me every day by Dr. Monod from the director of the Pasteur Institute, Dr. Trefouel, who personally signed it.

At the Pasteur Institute, I spent several months attempting to isolate the *lac* repressor without success. (Three years later, in 1966, Walter Gilbert and Benno Müller-Hill accomplished the isolation (17).) My subsequent efforts in Paris were devoted to the development of an experimental system for a new project I wanted to start after my return to NYU. After having studied the genetic code (*i.e.* the dictionary used in the translation of mRNA into protein), I wished to explore the mechanisms of translation, especially that of peptide chain elongation. Three elongation factors had just been identified by Lucas-Lenard and Lipmann (18). The obstacle toward further progress seemed to be that one of the three (designated as elongation factor EF-Ts) appeared to be unstable after purification.

I hoped that the thermophilic microorganism *Bacillus stearothermophilus*, growing at temperatures up to 70 °C, would be a source of factors stable for purification at lower temperatures (19). *B. stearothermophilus* turned out to be a good choice. With gloves to prevent burning one's fingers, *B. stearothermophilus* was convenient to work with: it had a generation time of <15 min, and at a temperature of growth of 70 °C, there was no need for sterility.

After a year in Paris, I returned to NYU as an assistant professor. At NYU, Israel Algranati, a visiting scientist from Argentina, joined me to continue the experiments with *B. stearothermophilus*. The thermal stability and high activity at 65 °C of the *B. stearothermophilus* system, as well as its low nuclease activity, allowed the translation at 65 °C of polynucleotides (*e.g.* 1.4:1 copolymer of adenylic and uridylic acids), which, because of their highly ordered secondary and tertiary structures, are almost inactive at 37 °C in an *E. coli* system (20).

Move to Yale

Studies on Peptide Chain Initiation and Elongation—In the fall of 1965, I joined the faculty of the Molecular Biophysics and Biochemistry Department at Yale. I believe that my invitation to the department by its chair, Fred Richards, might have originated from a series of conversations I had with Alan Garen from the department, among others, at several Cold Spring Harbor meetings and a meeting in Hyderabad, India. These conversations concerned the genetic code, a topic of interest for both of us.

My collaborators Art Skoultchi, Yasushi Ono, and Hong Mo Moon isolated, from the thermophilic

B. stearothermophilus, three elongation factors designated in the now accepted way: EF-Ts, EF-G, and EF-Tu (at the time, we designated them as S1, S2, and S3, respectively). All three factors were needed for the poly(U)-promoted formation of polyphenylalanine from Phe-tRNA (21). To make our studies more similar to those of Lucas-Lenard and Lipmann (18), we formed a ribosome·poly(U)·acetyl-Phe-tRNA complex *in vitro*. The acetyl-Phe-tRNA served as an analog of the natural chain initiator fMet-tRNA$_f$.

There were indications that an acetyl-Phe-tRNA· GTP·EF-Tu complex (ternary complex) can carry, in the presence of poly(U), Phe-tRNA to the ribosome. Ono *et al.* (22, 23) isolated such a complex and established that the molar ratio of GTP to Phe-tRNA was 1:1 and that EF-Tu can form a ternary complex with each aminoacyl-tRNA involved in chain elongation.

It was known that the chain initiator in *E. coli* is fMet-tRNA$_f$ synthesized by formylation of Met-tRNA$_f$. A different Met-tRNA (Met-tRNA$_m$) provides Met for internal positions of the peptide chain (24).

It was also known that as initiator codons in *E. coli*, GUG and AUG specify fMet-tRNA$_f$; as codons for internal amino acid residues, GUG stands for Val-tRNA, and AUG stands for Met-tRNA$_m$ (25). The dual specificity of GUG raised the question of how mix-ups between Val-tRNA, fMet-tRNA$_f$, and Met-tRNA$_f$ are avoided during chain elongation. As noted, aminoacyl-tRNA·GTP·EF-Tu complexes are intermediates in binding aminoacyl-tRNA to the ribosomes.

Ono *et al.* (26) established that EF-Tu and GTP do not form a complex with either Met-tRNA$_f$ or fMet-tRNA$_f$. This explains why Met residues are not inserted into the polypeptide chain in response to internal GUG codons.

We examined the fate of the three components of the ternary complex after the transfer of its Phe-tRNA moiety to the ribosome·poly(U)·acetyl-Phe-tRNA complex. We established that one GTP molecule was cleaved for each Phe-tRNA bound to the ribosome, and this cleavage was promoted by EF-Tu in the complex. Cleavage of an additional GTP is needed for translocation (23).

The GTP cleavage product P$_i$ was released in a free state, but the other, GDP, was released in complex with EF-Tu. Skoultchi *et al.* (27, 28) tested if GTP cleavage is a prerequisite for peptide bond formation in an experiment in which we substituted for GTP an analog, guanosine 5′-(β,γ-methylenetriphosphate) (GMPPCP), which has a methylene bridge instead of oxygen between the β- and γ-phosphorus atoms, so it cannot be cleaved enzymatically into GDP and P$_i$. In this case, equimolar

amounts of EF-Tu, Phe-tRNA, and GMPPCP were bound to the ribosome, but no dipeptide was formed. GTP cleavage is required for and has to precede the release of EF-Tu from the ribosome and peptide bond formation (27, 28).

In 1966, Eisenstadt and I proved that fMet-tRNA$_f$ is required for the translation of bacteriophage f2 RNA in an *E. coli* extract (29). The chain initiator codons AUG and GUG also specify internal amino acid residues. The characteristics that determine whether an AUG or GUG codon in an mRNA serves as a signal for initiating a new peptide chain were not known. Kondo *et al.* (30) developed a procedure for isolating a chain initiation signal containing segments of mRNA. It was known that the region of mRNA that is bound to ribosomes is protected against cleavage by nucleases. Furthermore, the intermediates in the assembly of a peptide chain initiation complex had been reported. First, a 30 S ribosomal subunit forms a complex with mRNA in the presence of GTP and initiation factors (31). Subsequently, the 50 S ribosomal subunit is attached, forming a 70 S initiation complex. We expected that the binding of mRNA in a 70 S initiation complex is dependent on fMet-tRNA$_f$, and if fMet-tRNA$_f$ is the only aminoacyl-tRNA present, the ribosome has to bind uniquely to the initiation signal (30).

We developed conditions in which bacteriophage f2 RNA did not become bound in a 70 S complex in the absence of fMet-tRNA$_f$. Moreover, in the presence of fMet-tRNA$_f$, only a single ribosome was attached to the f2 RNA molecule. Gupta *et al.* (32) isolated such a 70 S complex containing f2 RNA, treated it with ribonuclease, and recovered the 61-nucleotide-long segment of the f2 RNA that was protected against nuclease action by the attached ribosome. We presented only a partial sequence of this segment at a Cold Spring Harbor meeting. At the same meeting, Joan Steitz, who also used the approach of Kondo *et al.* (30) to obtain the initiation signal containing the segment of bacteriophage R17 RNA, presented its complete sequence, including the initiation signal (33). We presented the complete sequence of the initiation signal containing the segment of f2 RNA subsequently (34).

Movement of the Ribosome along Bacteriophage f2 mRNA during Protein Synthesis—Having established the conditions for attaching a ribosome to a single initiation signal on an mRNA enabled us to study the movement of the ribosome during peptide chain elongation. Gupta *et al.* (35) determined that (*a*) one step of the ribosome along the mRNA is (as expected) 3 nucleotides long, and (*b*) the movement of the ribosome along the mRNA during peptide chain elongation is triggered by EF-G and

GTP. The experiments on which these conclusions were based included the following. A single ribosome was bound to bacteriophage f2 RNA in the presence of fMet-tRNA$_f$ under conditions in which it is bound to the coat protein initiation site (initiation complex (IC)). An aliquot of the IC was converted into a pre-translocation complex (PRC) in a reaction with EF-Tu, GTP, and Ala-tRNA. (Ala is the second amino acid of the coat protein.) In the PRC, the fMet-Ala-tRNA is bound at the A-site, and an uncharged tRNA is bound at the P-site of the ribosome. An aliquot of the PRC was converted into a post-translocation complex (POC) in a reaction with EF-G and GTP. In the POC, the fMet-Ala-tRNA is bound at the P-site of the ribosome, and the A-site is vacant. Each of the three complexes was treated with ribonuclease to digest those parts of the f2 RNA not protected by the attached ribosome and then centrifuged to separate the ribosomes with the protected f2 RNA fragment from other components.

Each of the three isolated ribosome·f2 RNA complexes was examined in two ways. First, the f2 RNA fragments were isolated from each, and the nucleotide sequences at the 3′-terminal region of the fragments were determined. The main f2 RNA fragment in the POC (3′-UUUACU) extended 3 nucleotides farther toward the 3′-end than that present in both in the IC and PRC (3′-UUU). Second, each ribosome·f2 RNA fragment complex was suspended in a reaction mixture including labeled amino acids, and the f2 RNA fragment in it was translated into oligopeptides. The oligopeptides from the POC had a C-terminal Thr residue (corresponding to -UUUACU), whereas those from the IC and PRC had C-terminal Phe residues (corresponding to -UUU) (35).

The above conclusions about ribosome movement are based on the difference between the 3′-ends of the mRNA fragments protected against RNase cleavage in the IC, PRC, and POC. Thus, they reflect the movement along the mRNA of those parts of the ribosomes that protect the mRNA.

I reviewed the mechanisms of protein synthesis in a survey written with Dieter Söll (36) and also in the summary of the 1969 Cold Spring Harbor Symposium on the Mechanism of Protein Synthesis (Fig. 3) (37).

The Second Genetic Code: Recognition of Two Serine tRNAs Specific for Unrelated Codons by the Same Seryl-tRNA Synthetase

By 1966, the bulk of the genetic code used in mRNA to specify amino acids had been deciphered. However, the problem of what might be called the second genetic

FIGURE 3. **Peter Lengyel (East Greenwich, Rhode Island, 2007).**

code (*i.e.* the signals by which aminoacyl-tRNA synthetases recognize their specific tRNA) was hardly touched (38).

Each tRNA has a specific region whose nucleotide sequence is complementary to those of the codons specifying the aminoacyl residue attached to the tRNA. This region is designated as the anticodon. Each aminoacyl-tRNA synthetase is specific for one amino acid. It was an obvious question of whether the anticodon is also the specific recognition site for the aminoacyl-tRNA synthetase. The answer to this question was different in various publications.

To clarify this problem, G. Sundharadas and I, in collaboration with the laboratories of Dieter Söll and William Konigsberg at Yale, made use of two tRNA^{Ser} species in *E. coli* that respond to codons with entirely unrelated nucleotide sequences: tRNA^{Ser} IIa to AGU and AGC and tRNA^{Ser} IIb to UCA and UCG (39). The two tRNA^{Ser} species in question were described by Söll *et al.* (40).

We purified and characterized Ser-tRNA synthetase from *E. coli*, as well as the two tRNA^{Ser} species with unrelated anticodon sequences. These two tRNA^{Ser} species competed with each other in charging by the purified Ser-tRNA synthetase. The fact that the same enzyme recognized the two tRNA^{Ser} species with entirely unrelated anticodons indicated that the anticodon cannot be the site of specific recognition for Ser-tRNA synthetase. This revealed that the so-called second genetic code, which deals with the recognition of tRNA by aminoacyl-tRNA synthetase, is different from the genetic code that concerns the translation of codons in mRNA into amino acids (39).

Exploring the Interferon System

In 1966, when I explored aspects of the mechanism of protein synthesis, I noticed a paper by Phil Marcus from the Albert Einstein College of Medicine of Yeshiva University concerning a topic novel to me: the mode of action of interferon (IFN), an agent whose designation was based on its ability to interfere with the replication of viruses. Based on their findings, Marcus and Salb (41) hypothesized that IFN inhibits viral protein synthesis. I found their hypothesis intriguing. When I joined the small field of IFN research, no IFN had yet been purified. Many people even doubted it existed, and some called it "misinterpreton." In 1972, following others, we started to explore the inhibition of reovirus replication by IFN. Ahmad Vassef and others in our laboratory demonstrated the selectivity of IFN action showing that concentrations of IFN that inhibited the replication of vesicular stomatitis virus and reovirus in cells did not affect the hormonal induction of the protein-tyrosine aminotransferase in cells (42).

Richard Galster in my laboratory obtained intriguing results in 1976. IFN treatment of cells gave rise to subviral particles with an associated endonuclease and produced shorter reovirus RNAs than subviral particles from control cells *in vitro*. The endonuclease cleaved added ribosomal RNA *in vitro*, suggesting that the shorter reovirus RNAs were the results of enzymatic cleavage (43).

Brown *et al.* (44) found that an extract from cells treated with an IFN preparation or with the IFN inducer poly(I)·poly(C) (which we designated as S30$_{INT}$) degraded reovirus mRNAs faster than an extract from control cells (S30$_C$). The reovirus mRNA preparation we used was contaminated with reovirus genomic double-stranded RNA (dsRNA). After removing the contaminating dsRNA and repeating the experiment with the dsRNA-free reovirus mRNA, we were dismayed to find no difference between S30$_{INT}$ and S30$_C$ in the rate of cleavage of reovirus mRNA. The addition of reovirus dsRNA or the synthetic dsRNA poly(I)·poly(C) accelerated the degradation of reovirus mRNA in S30$_{INT}$ but not in S30$_C$. We tentatively designated this novel IFN-inducible endonuclease requiring dsRNA for promoting the cleavage of mRNA in S30$_{INT}$ as endonuclease$_{INT}$. In 1979, after having characterized the activation of endonuclease$_{INT}$ (44), we changed its designation to RNase L (with L standing for latent) (45).

Sen *et al.* (46) found that ATP was required, in addition to dsRNA, to promote RNA cleavage in S30$_{INT}$. In step 1 (endonuclease$_{INT}$ activation), dsRNA and ATP were

FIGURE 4. **Ganes Sen (Cleveland, 2004).**

required (but not reovirus mRNA). In step 2 (endonuclease action), in which neither dsRNA nor ATP was required, added reovirus mRNA was degraded (Fig. 4) (46). We first hypothesized that activation of endonuclease$_{INT}$ might be due to phosphorylation by a protein kinase induced by IFN and activated by dsRNA for which ATP served as a phosphate donor.

However, our further exploration of the nature of the agent-activating endonuclease$_{INT}$ (*i.e.* RNase L) revealed that this hypothesis was wrong. In 1978, Ratner *et al.* (47) established that the incubation of dsRNA with S30$_{INT}$ triggered the formation of a small thermostable molecule that, when added to S30$_{INT}$ (but not to S30$_C$), activated the latent endonuclease$_{INT}$ (*i.e.* RNase L). To cite Ratner *et al.* (47), "the small thermostable molecule in question shares the following characteristics with a low molecular weight, heat-stable component reported by Kerr *et al.* (1977) [48], synthesized in S30$_{INT}$ in the presence of dsRNA and ATP: it is inactivated by snake venom diesterase, but not by P1 RNase." The discovery of this heat-stable compound inhibiting cell-free protein synthesis and its identification as 2′-5′ oligoadenylate (2–5A) were reported by Kerr *et al.* (48) and by Kerr and Brown (49) when aiming to identify the mediator of the greater sensitivity to dsRNA of protein synthesis in S30$_{INT}$ than in S30$_C$. The addition of 2–5A to S30$_{INT}$ triggered an increase in endonuclease activity. Baglioni *et al.* (50) and Clemens and Williams (51) con-cluded that this increase is consistent with earlier reports from our laboratory (44, 46). These indicated that endonuclease$_{INT}$ (44, 46), which was renamed RNase L in 1979 (45), and 2–5A (48, 49) are two components of the IFN-inducible, dsRNA-activated endonuclease system.

Farrell *et al.* (52) established that there are two distinct pathways by which dsRNA inhibits protein synthesis (see Fig. 5 in Ref. 52). The first (pathway a) is described in some detail at the end of the previous paragraph: it consists of endonuclease$_{INT}$ (44, 46), renamed RNase L (45), and 2–5A (52, 53). The second (pathway b) is a purified, IFN-induced protein kinase (53) that, if activated by dsRNA and ATP, will phosphorylate and thereby inactivate the small subunit of the peptide chain initiation factor eIF-2.

Slattery *et al.* (45) established that removal of 2–5A from RNase L restored its latency. The 2–5A RNase L system is a mediator of the inhibition of encephalomyocarditis virus replication by IFN (54).

Before discontinuing our work on the 2–5A/RNase L system, we characterized its RNA cleavage pattern. Using partially purified RNase L, Georgia Floyd-Smith and Elizabeth Slattery established that, among the homopolyribonucleotides poly(A), poly(C), poly(G), and poly(U), activated RNase L cleaved only poly(U) at an appreciable rate. In two natural RNAs tested, the most frequent RNase L cleavages occurred after UA, UG, and UU sequences, and much less frequent cleavages occurred after CA and AC sequences (55, 56).

Gribaudo *et al.* (57) noted that, in HeLa cells, 2–5A synthetase can be in a complex including both strands of encephalomyocarditis virus RNA. St. Laurent *et al.* (58) found that IFN-treated Ehrlich ascites tumor cells contain at least two distinct 2–5A synthetases translated from at least two distinct mRNAs.

After revealing the RNA cleavage specificity of the 2–5A RNase L system (55) in 1981, we shifted our efforts to the characterization of the various activities of the IFN system. From that time on, the bulk of the progress in understanding the 2–5A RNase L system was generated in the laboratory of R. H. Silverman and his collaborators: Zhou (59, 60), Malathi (61), and Zhao (62).

Purification of Three Murine IFN Species to Homogeneity—Cabrer *et al.* (63) purified mouse IFNs from Ehrlich ascites tumor cells infected by Newcastle disease virus. This resulted in three homogeneous protein bands. The first N-terminal sequences of the three murine IFN species (one α type and two β types) were reported by Taira *et al.* (64) in the same issue of *Science* as those of the first N-terminal sequences of human lymphoblastoid IFN and human fibroblast IFN.

FIGURE 5. **Paul Farrell (London, 2014).**

García-Blanco *et al.* (65) demonstrated that even within the same cells, the IFN system can discriminate between the expression of the SV40 T-antigen from an infecting viral genome (which it inhibits) and the expression of the same SV40 T-antigen in the integrated SV40 viral genome (which it does not affect).

IFN Induces Expression of New mRNA and New Protein— The availability of pure mouse IFN (63, 64) and the finding that inhibition of RNA or protein synthesis blocks the establishment of the antiviral state by IFN prompted Farrell *et al.* (66) to search for new mRNAs and the proteins they encode in cells treated with pure IFN. *In vitro* translation of mRNA from Ehrlich ascites tumor cells treated with IFN resulted in 5-fold more of a M_r 14,500 protein than translation of mRNA from control cells. Moreover, 7.5-fold more of the same protein was found in an extract from IFN-treated cells than in that from control cells. This revealed that IFN can induce particular proteins, and this may be due to increasing the level of particular mRNAs (Fig. 5) (66, 67). The M_r 14,500 protein was later identified as diubiquitin (68). The induction of an M_r 56,000 protein in chick cells by IFN was also reported by Ball (69) in 1979.

Cloning cDNAs Complementary to IFN-induced mRNAs— The above findings of Farrell *et al.* (66) were the basis of an experiment by Samanta *et al.* (70) in 1982. They gen-

erated labeled cDNAs from RNA isolated from IFN-treated cells. From this labeled cDNA preparation, they removed the molecules complementary to mRNAs also present in control cells. The resulting labeled cDNA was used to identify IFN-inducible mRNAs. The first one of these novel mRNAs (designated as 202 mRNA) served as the starting material for a new project concerning the IFN-inducible proteins of the multifunctional p200 protein family (see "Exploration of the Biological Roles of the Multifunctional p200 Family Proteins" below) (70).

A Stroll in the Woods with Charles Weissmann

Charles and I were colleagues in the Ochoa laboratory. Later, we kept in touch and informed each other about our research at least once a year. One of these exchanges occurred at a Gordon Conference in the summer of 1977 when strolling in the woods. This stroll, as described by Charles in an informative paper entitled "The Cloning of Interferon and Other Mistakes" (71), had important effects on IFN research. I told Charles about our studies of IFN action and the difficulties of obtaining pure IFN (this conversation took place before we completed the purification in 1980) (64). I mentioned the existence of a convenient assay for IFN mRNA based on its injection and translation in *Xenopus* oocytes (72). Charles had and I lacked experience in cloning. He had an idea how to clone an IFN cDNA, and we decided to collaborate in the cloning of a mouse IFN cDNA. I asked H. Taira, who worked on IFN production and purification in our laboratory, to join Charles's team at the University of Zurich. Sometime later, Charles accepted an invitation to join the founding scientific board of Biogen. This prompted him to shift his efforts in IFN cloning from mouse to human IFN. The rest of this story is history: it resulted in the first cloning of a human IFN by Charles and his co-workers. A few years thereafter, Charles sent Werner Boll, one of his associates, to my laboratory to clone a murine IFN (73).

The Murine *Ifi200* Gene Cluster and the Multifunctional p200 Protein Family

The finding that IFN treatment can activate the expression of genes (66) prompted us to clone IFN-activatable genes. As noted above, the first such murine cDNA clone (*Ifi202* cDNA) encoded 202 mRNA (70). The gene encoding this mRNA was mapped to chromosome 1. Chromosomal walking by Engel *et al.* (74) and restriction enzyme mapping, hybridization analysis of cosmids, and sequencing by Opdenakker *et al.* (75) and Choubey *et al.* (76) revealed the existence of at least six closely linked, structurally related, and IFN-activatable murine genes. These

include, among others, *Ifi201*, *Ifi202*, three *Ifi203*-type genes, and *Ifi204*. They are parts of the murine *200* family gene cluster. This is located within a 100-kb segment between the *Spna1* and *Sap* loci on chromosome 1 (76). Further sequencing of two long DNA segments by Deschamps *et al.* (77) revealed the existence of additional p200 family genes in the cluster.

A comparison of the p204 and p202 proteins from the cluster revealed that as much as 47% of their amino acid sequences is conserved. Further sequencing studies suggested that the evolution of the cluster involved duplication of a DNA segment, generating a double-length transcription unit. A further duplication and divergence of this unit gave rise to the two IFN-activatable *202* and *204* genes (76).

Analysis of the sequences of the various *200* family genes by Choubey *et al.* (76) revealed, in each, one or two partially conserved 600-nucleotide segment(s) encoding 200 amino acid domains (also designated as HIN domains). These were classified first into A- and B-types according to sequence, and thereafter, a C-type was added.

The human *IFI200* (or *HIN*) gene cluster, whose first member, *IFI16*, was discovered by Trapani *et al.* (78) in 1992, also contains the *MNDA*, *AIM2*, and *IFIX* genes. Ludlow *et al.* (79) published a review concerning the p200 family genes and proteins.

The evolution of the *Ifi200* (and *IFI*200) gene clusters in several species involved repeated gene duplications. This contributes to the large differences in the sequences of the clusters in various mouse strains (80).

In placental mammals, there is one *Ifi200* gene in the cow and up to 14 in different strains of mice; in each of three marsupials (kangaroo, opossum, and bandicoot), there is one *Ifi200* gene. Unexpectedly and remarkably, Cridland *et al.* (80) did not find any *Ifi200* (*PYHIN*) family genes in non-mammals.

Exploration of the Biological Roles of the Multifunctional p200 Family Proteins

Most of the earlier biological research was initiated by the observation of a biological or biochemical process and was continued by the identification of the agent(s) responsible and their modes of action. In the case of p202 and p204 (the earliest studied p200 family proteins), the cDNAs were cloned first, and only thereafter was the search started for the functions of the encoded proteins (81).

p202 as a Modulator of Transcription

The p202 protein is IFN-inducible in cell lines from several inbred strains of mice, and it is phosphorylated. After its induction by IFN, p202 accumulates first in the cytoplasm and moves to the nucleus only after a delay. In metaphase cells, p202 appears to be associated with chromatin. p202 extracted from IFN-treated cells binds dsDNA *in vitro*. Overexpression of p202 inhibits growth (82). The recognition of the binding to p202 of the first growth regulatory protein, pRb (encoded by the retinoblastoma tumor suppressor gene), was based on the presence of the L*XCX*E sequence in p202. This sequence is present in several pRb-binding proteins (82).

As revealed by the studies of Choubey *et al.* (83), Min *et al.* (84), and Wang *et al.* (85), inhibition of cell growth by p202 may be correlated with its binding and inhibition of the activity of several transcription factors, including E2F-1, E2F-4, c-Fos, c-Jun, MyoD, myogenin, c-Myc, and NF-κB (83–85). In most cases, p202 inhibits the sequence-specific binding of the transcription factor to DNA. As found by Ma *et al.* (86), the effects of p202a on the activities of the NF-κB transcription factor and its subunits, p50 and p65, are complex: p202a inhibits the binding to DNA of p50/p65 heterodimers and p65 homodimers (both of these complexes activate transcription) while enhancing the binding of p50 homodimers inhibiting transcription. Datta *et al.* (87) established that the level of p202 increases during the fusion of cultured myoblasts to myotubes. p202 inhibits the activation of transcription by p53, and a segment from 53BP1 (p53-binding protein 1) overcomes this inhibition (87).

The human adenovirus 5-encoded E1A protein promotes cell proliferation. It does this through the targeted interaction with cellular proteins that act as negative regulators of cell growth. The targets of E1A (besides pRb) include p202. E1A protein overcomes the p202-mediated inhibition of cell growth. As reported by Xin *et al.* (88) from the Choubey laboratory, this is the consequence of alleviation of a p202-mediated inhibition of the activity of the E2F-1 transcription factor: E1A binds p202 and reduces its binding to E2F-1.

p202 is also anti-apoptotic (88, 89). This effect is at least in part the consequence of binding and inhibiting the activity of the pro-apoptotic proteins p53 (87) and c-Myc (85). By inhibiting the activity of the various transcription factors listed (and likely also other ones) and, as noted, being growth inhibitory, p202 is expected to affect many biological processes. Thus, p202 was reported to be a mediator of chondrogenic and osteogenic differentiation (90).

Knocking out a p202 Gene—To study the function(s) of the p202 gene, in 1999, Wang *et al.* (91) knocked it out in mice. Unexpectedly, the study revealed the existence of two novel murine p202 genes (*Ifi202b* and *Ifi202c*). Both

are close homologs of the first described *Ifi202* gene (which we renamed *Ifi202a*). The three *202* genes colocalize to chromosome 1. *Ifi202b* is IFN-inducible. It differs from *Ifi202a* in 7 amino acids. *Ifi202c* is an unexpressed pseudogene. The *Ifi202* gene that we knocked out from strain 129 mice was *Ifi202a*. The expression of Ifi202b mRNA (but not 202a mRNA) persisted in the knock-out mice at the same level as in wild-type mice. However, in murine embryonic fibroblasts from the knock-out mice, the constitutive and IFN-induced levels of p202b were approximately as high as those of p202a and p202b in murine embryonic fibroblasts from wild-type mice. This suggested dosage compensation at the post-transcriptional levels. The latter might account for the apparent lack of phenotype of the knock-out mice.

Recognition of Intracellular Viral DNA by p202 Protein and Inhibition by p202 of the Antiviral Activity of AIM2 Protein—p202 in an extract from IFN-treated AKR-2B cells was found to be retained on a dsDNA-cellulose column in 1993 (81), as was purified GST-202 in 1996 (92). Recently, Roberts *et al.* (93) reported that the binding of p202 to dsDNA from a virus can be a modulator of the antiviral activity of AIM2. The latter is a p200 family protein in mice that has a human counterpart with the same name. (Although the above activity of p202 was discovered after I had closed my laboratory, I mention it here because of its importance.) AIM2 also binds dsDNA in the cytoplasm. Moreover, the AIM2-DNA complex initiates the pathway generating inflammasomes and thereby triggers the death of virus-infected cells (93, 94). p202 antagonizes the generation of inflammasomes by AIM2: the p202 HIN2 domain binds the AIM2 HIN2 domain, thereby inhibiting the clustering of AIM2 on dsDNA (95). However, the generation of inflammasomes and of mature proinflammatory cytokines killing the virus-infected cells does require such clustering (93–95).

p204, a Multifunctional Modulator of Proliferation and Differentiation

p204 Inhibits Ribosomal RNA Synthesis—p204 was found to be nucleolar and nucleoplasmic in cultured murine cells (96). In the nucleoli and also in a cell-free system, p204 was shown by Liu *et al.* (97) to inhibit ribosomal RNA synthesis. The inhibition was overcome *in vitro* by the addition of the ribosomal RNA-specific transcription factor UBF1 (upstream binding factor-1). p204 bound UBF1, and it inhibited the binding of UBF1 to the regulatory region of the ribosomal DNA genes (97). p204, which, like p202, contains L*X*C*X*E sequences,

bound the three pocket proteins pRb, p107, and p130 (98).[1]

Involvement of p204 in Skeletal Muscle Myotube Differentiation—A comparison of the levels of p204 protein in 10 tissues of adult mice by Liu *et al.* (99) revealed the highest p204 levels in the heart, skeletal muscle, and kidney. In cultured C2C12 skeletal muscle myoblasts, p204 was nucleoplasmic, and its level was low. During the differentiation (*i.e.* the fusion of myoblasts to myotubes), this level strongly increased, p204 became phosphorylated, and the bulk of it appeared in the cytoplasm of the myotubes. Leptomycin B, an inhibitor of nuclear export, blocked this translocation to the cytoplasm and also inhibited myoblast fusion. The increase in the p204 level during differentiation was a consequence of the binding of MyoD, myogenin, and E12/E47 transcription factors to several MyoD-specific sequences in the p204 gene, followed by transcription. Overexpression of p204 in C2C12 myoblasts accelerated their fusion to myotubes. A decrease in the p204 level in the C2C12 myoblasts by antisense RNA inhibited the fusion in differentiation medium (100).

Id (inhibitor of differentiation) proteins inhibit muscle differentiation by binding and inhibiting the binding to DNA of MyoD and other basic helix-loop-helix (bHLH) transcription factors (101, 102). Liu *et al.* (100) found that p204 bound to Id proteins, blocked their binding to bHLH transcription factors (*e.g.* MyoD, myogenin, and E12/E47), and thereby overcame their inhibition of bHLH transcription factor activity and muscle differentiation (Fig. 6).

Involvement of p204 in the Differentiation of P19 Stem Cells to Beating Cardiac Myocyte-type Cells—The high level of p204 in adult mouse heart muscle and also in isolated neonatal cardiac myocytes prompted Ding *et al.* (103) to explore whether p204 is involved in cardiac myocyte differentiation. As a test system, we used cultured P19 murine embryonal carcinoma stem cells, which were reported to be triggered to differentiate to beating cardiac myocyte-type cells by dimethyl sulfoxide (104). Dimethyl sulfoxide induced p204 RNA and p204 formation (103). Ectopic p204 induced differentiation, whereas ectopic 204 antisense RNA inhibited the differentiation induced by dimethyl sulfoxide. Experiments with reporter constructs including the regulatory region from the *Ifi204* gene in P19 cells and in cultured newborn rat cardiac myocytes revealed that p204 expression was synergistically transactivated by the cardiac Gata4,

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[1] C. Liu and P. Lengyel, unpublished data.

FIGURE 6. **Chuanju Liu (New York City, 2014).**

Nkx2.5, and Tbx5 transcription factors (103). The regulatory region of the *Ifi204* gene contains several binding sites for these three factors. In turn, ectopic p204 triggered the expression of Gata4 and Nkx2.5 in P19 cells. p204 contains a nuclear export signal and was partially translocated to the cytoplasm during differentiation. p204 in which the nuclear export signal was mutated was not translocated, and it did not induce differentiation (103).

Endogenous or ectopic Id protein inhibited the differentiation of P19 embryonal carcinoma stem cells to beating cardiac myocyte-type cells (105). This was the consequence of the binding of Id1, Id2, or Id3 protein to the Gata4 and Nkx2.5 proteins and the resulting inhibition of (*a*) the binding of these transcription factors to each other and to DNA and (*b*) the synergistic transactivation of the expression of various genes, including *Ifi204*.

Ding *et al.* (105) also determined that p204 overcame this inhibition of transcription factor activity by Id proteins as a consequence of (*a*) binding and sequestering Id proteins, (*b*) decreasing the level of Id proteins in the nucleus by increasing their translocation from the nucleus to the cytoplasm, and (*c*) accelerating the ubiquitination of Id proteins and their degradation by proteasomes, with the last two activities dependent on the presence of the nuclear export signal in p204. In the course of the differentiation, Gata4, Nkx2.5, and p204 were components of a positive feedback loop: Gata4 and Nkx2.5 promoted p204 expression, and p204 overcame the inhibition of the synergistic activity of Gata4 and Nkx2.5 by the Id proteins (105).

Expression of p204 in Several Organs of Mice Does Not Depend on IFN—Although p202 and p204 were discovered as members of a protein family inducible by IFN, Wang *et al.* (106) established that the levels of p202 and p204 in several tissues were the same in wild-type mice as in mice lacking IFN receptors. This indicated that p202 and p204 can be expressed independently of IFN. Indeed, the expression of p204 in the course of the differentiation of skeletal muscle and heart muscle is independent of IFN and is promoted by muscle-specific and cardiac transcription factors, respectively (99, 103).

p204 as a Modulator and Feedback Inhibitor of Wild-type Ras Protein and Ras Oncoprotein Activity—The findings that p204 is translocated to the cytoplasm during skeletal and cardiac muscle differentiation and that most of p204 is cytoplasmic in mouse heart and in isolated cardiac myocytes (99, 103) prompted a search by Ding and Lengyel (107) for cytoplasmic proteins in the heart that are bound by p204. A yeast two-hybrid assay showed that K-Ras protein was among these. p204 bound endogenous or ectopic K-Ras and its sister protein, H-Ras, in cell lysates. Purified p204 bound *in vitro* both purified K-Ras and H-Ras, which are encoded by the K-*ras* and H-*ras* genes. Particular mutants of these two genes, *i.e.* K-*ras* and H-*ras* oncogenes, play a causal role in >20% of human cancers (108). K-Ras and H-Ras proteins cycle between an activated and an inactivated form, Ras-GTP and Ras-GDP. Ras proteins control signaling pathways regulating transcription, translation, cell growth, cell shape, apoptosis, senescence, and malignant transformation (109). Ras oncoproteins are persistently activated because, unlike wild-type Ras proteins, they are unable to promote the cleavage of the bound GTP to GDP. This cleavage is promoted in the case of wild-type Ras protein by Ras GTPase-activating protein binding to Ras-GTP. There are numerous mediators of Ras signaling, which are designated as Ras effectors. They bind to Ras-GTP and initiate numerous signaling pathways. The three effectors first identified were Raf-1 (a serine/threonine kinase), PI3K, and Ral-GDS, a GDP-GTP exchange factor for Ral proteins (108, 109).

Ding and I (107) established that purified p204 bound to purified H-Ras- or K-Ras-GTP; inhibited their cleavage to Ras-GDP by Ras GTPase-activating protein; and inhibited the binding to H-Ras- and K-Ras-GTP of Raf-1, PI3K, and Ral-GDS, Ras effector proteins that initiate three of the signaling pathways by wild-type Ras protein or oncoprotein. This inhibition caused inhibition of activation by phosphorylation of some downstream targets of Ras (*e.g.* MEK, Akt, and p38 MAPK).

Oncogenic *ras* expression triggered the phosphorylation and translocation of p204 from the nucleus to the cytoplasm, the compartment in which Ras is also located. The translocation was blocked by inhibiting PI3K activity. p204 inhibited the anchorage-independent proliferation of cells expressing Ras oncoprotein and also blocked the rearrangement of the actin cytoskeleton and the enhancement of cell migration through a membrane by Ras oncoprotein (107).

Ras oncoprotein and wild-type Ras-GTP were reported to increase the expression of the Egr-1 transcription factor (110, 111). We found that Ras oncoprotein or an increase in the wild-type Ras-GTP level increased the level of p204, together with that of Egr-1, as a consequence of the boost in transcription of 204 mRNA by Egr-1 (107). The probable significance of p204 as a tumor suppressor was revealed by finding that activation of the expression of a single copy of the K-*ras* oncogene in murine embryonic cells was sufficient to induce the high level expression of p204 and its distribution between the nuclei and the cytoplasm (107). Thus, p204 can serve as a negative feedback inhibitor of Ras activity. It should be noted that an increase in the level of p202 induced in NIH 3T3 cells by the expression of oncogenic H-*ras* was reported by Xin *et al.* (112).

An additional nuclear activity of p204 in promoting differentiation was reported by Luan *et al.* (113). p204 binds pRb (as noted above), and the resulting complex binds and increases the transcriptional activity of the Cbfa1 transcription factor involved in osteoblast differentiation.

Parting Thoughts Concerning p202 and p204

As noted in the preceding section, p204 is a modulator and feedback inhibitor of Ras oncoprotein activity (107). It remains to be established if p204 is beneficial in the defense against Ras oncoprotein-promoted tumors.

Some of the p200 family proteins in mouse (*e.g.* p202 and p204) are highly multifunctional (114, 115). Therefore, as noted above, it was unexpected that no *Ifi200* family genes were found by Cridland *et al.* (80) in non-mammals (except a single such gene in each of three marsupials (kangaroo, opossum, and bandicoot)). The multifunctionality of p202 and p204 in the mouse and probably of IFI16 (the closest homolog of Ifi204) in humans makes one wonder if their knock-out could be tolerated under particularly stressful conditions.

The successful knock-out of a murine p202 gene (p202a) was reported (91). However, it turned out that p202a has an active sister gene (p202b) that can substitute for it. Furthermore, as noted, the knock-out of p202a increased the expression of p202b, apparently, at the post-transcriptional level.

Further exploration of the functions of the multifunctional p202 and p204 proteins could be facilitated by generating mice in which p202 or p204 formation could be inhibited by inducible tissue-specific or, possibly, embryo developmental stage-specific knock-out (116).

Thanks

After leaving NYU and spending five months at the Cold Spring Harbor Laboratory, I moved to the Department of Molecular Biophysics and Biochemistry at Yale in the fall of 1965. During my long stay here, I continually valued and liked the institution; the high quality of its faculty and students; and the generally humane, democratic, and informal tradition of the university.

At Yale, my research depended on my collaborators (undergraduates, graduate students, M.D.-Ph.D. students, and postdoctoral associates coming from various parts of the world), many of them highly motivated and enthusiastic. Altogether, I had about a hundred collaborators during the forty-two years I maintained my laboratory at Yale.

I remain grateful to them for making our work possible. It has been a joy to follow their careers both in academia and in industry. I am very pleased that many lifelong friendships developed among them.

I taught a variety of courses through my years at Yale. I invested perhaps the most effort in and benefited the most from a course for those first-year medical students who, being well prepared in biochemistry, volunteered to take the course. We assumed they had the basic information, so we attempted to include recent developments. It was a demanding set of lectures to learn from and also to prepare.

I very much appreciate that I have remained in touch with some of my former collaborators. I thank my colleagues in the Department of Molecular Biophysics and Biochemistry for advice and help during my close to 50 years at Yale.

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Address correspondence to: peter.lengyel@yale.edu.

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